Z. Klin. Chem. Klin. Biochem.
10. Jg. 1972, S. 509—511

Influence of pH on the Extraction of Gonadotropic Hormones from Human Urine

By E. Finné, A. Van Humskerken and R. Vanden Driessche

Laboratory of Pharmacology, University of Brussels, Belgium

(Eingegangen am 9. April/4. Juli 1972)

The human gonadotropic hormones can be precipitated from urine with alcohol. The precipitate is then extracted with an aqueous buffer. The influence of pH on this extraction procedure was studied with respect to the differences in the results obtained by biological and chemical assay, i. e. augmentation of the mouse uterine weight and the determination of sialic acid.

Die menschlichen gonadotropen Hormone können nach Fällung mit Alkohol aus dem Harn extrahiert werden. Der Niederschlag wird mit einer wäßrigen Lösung extrahiert. Wir haben den Einfluß des pH dieser wäßrigen Lösung mit dem Ziel studiert, dabei den Unterschied zwischen den Resultaten der biologischen und chemischen Bestimmungen, das heißt, der Gewichtserhöhung des Mäuseuterus und der Bestimmung der Sialinsäure zu beobachten.

In earlier investigations one of us proposed the estimation of the total gonadotropic activity in urinary extracts by the determination of their sialic acid content (2—6). A satisfactory correlation was found between this chemical method and the biological method according to Klinefelter (augmentation of the uterus weight of immature mice (1)).
In this investigation, we have studied the effect of pH on the extraction of the alcohol precipitate of human urine, by determining the gonadotrophins both biologically and chemically.

Methods

Preparation of extracts (1)

Human male urines were collected, acidified to pH 4.5 with acetic acid and precipitated with 3 volumes ethanol 95°C. After standing at 4°C for 24 h, the precipitate was removed by filtration on a Büchner funnel and washed with acetone.
The precipitate was dried in a dessicator in vacuo at 4°C for 24 h and powdered ("crude extract").

Preparation of purified extract

Centrifugal purification

2 g crude extract were mixed for 20 min with 8 ml of a Na_2HPO_4 (0.2 mol/l)-citric acid (0.1 mol/l) buffer (8) at different pH values.
After centrifugation at 2000 rpm at 4°C for 15 min, the supernatant fluid was collected. The precipitate was washed twice with 5 ml of the appropriate buffer and recentrifuged. The supernatants were pooled and dialyzed for 20 h against distilled water at 4°C. The dialysates were lyophilised. The lyophilised powders are called "purified extracts".
The sialic acid content was measured after each step of the purification.

Column extraction

A glass column (1 × 10 cm), with a reservoir on the top, was filled with 1 g crude extract, which was eluted with 40 ml of the buffered solution used above, at different pH's. The following steps were the same as in the centrifugal extraction.

The essential difference between these two extraction methods is: with the centrifugation, the extraction time of the substrate is relatively short (ca. 1 h), whereas with the column method, this time is much longer (ca. 10—12 h).
Furthermore, the variation of pH of the extraction fluid is greater with the centrifugal procedure. For example the pH at the beginning of the extraction with centrifugation was 8.0 and at the end 6.65.
With column extraction, starting with pH 8.1, the final pH was still 8.1.

Concentration of sialic acid

An aliquot of 50 mg extract or 2 ml extract in solution were taken and the sialic acid content determinated by the resorcine method of Svennerholm (7).

Biological test

Immature female mice 18 days old (C. E. A. L. 72 — Ardenay — France) were injected with a quantity of extract, corresponding to 350 µg sialic acid. The animals received 1 daily intra-peritoneal injection for 3 days and were autopsied on the fourth day.
The animals were killed with ether vapor and both uterine-horns removed and weighed (± 0.1 mg).

Results

Centrifugal purification

By using the above mentioned method, we obtained the results summarised in Table 1 (chemical determination) and in the Table 2 (biological determination).

Tab. 1
Centrifugal purification, results of the chemical determination

Extraction No.	pH at the end of extraction	Total sialic acid content [µg]	Sialic acid content of the purified extract [µg]
I	3.55	8.400	5.793
II	4.5	8.400	6.512
III	5.4	8.400	7.123
IV	5.6	8.400	5.745
V	6.2	8.400	5.821
VI	6.65	8.400	5.462

Tab. 2
Centrifugal purification, results of the biological determinations

	Zero test	I	II	III	IV	V	VI
pH		3.55	4.5	5.4	5.6	6.2	6.65
mean uterus weight/10 g animal weight [mg]	6.6 ± 0.6	13.4 ± 1.2	16.5 ± 1.2	10.9 ± 0.8	12.1 ± 1.3	13.4 ± 0.9	15.4 ± 2.0
% augmentation		101.6	149.6	64.7	82.6	101.9	132.4

The total sialic acid content in the table corresponds to the quantity of sialic acid contained in 2 g crude extract and for this reason was the same at the start of each extraction.

The results of the two determinations, i. e. chemical and biological are compared in Figures 1 and 2.

Figure 1 shows the results when using the final pH; Figure 2 gives the results of the same experiment, but the pH values are those of starting buffer.

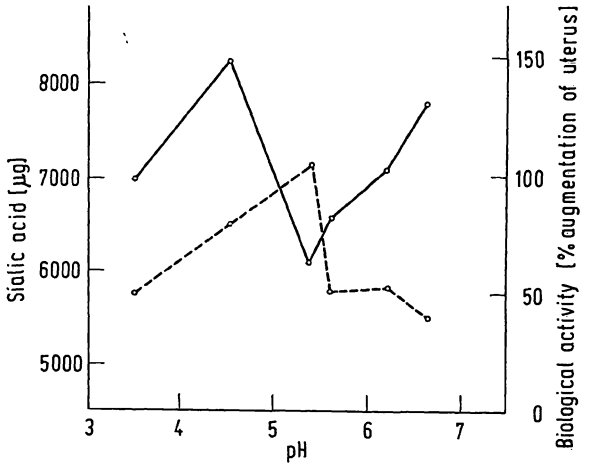

Fig. 1
Biological activity in % augmentation of the uterus weight of immature mice (— o —) and sialic acid content (- - - o - - -) versus pH by centrifugal extraction

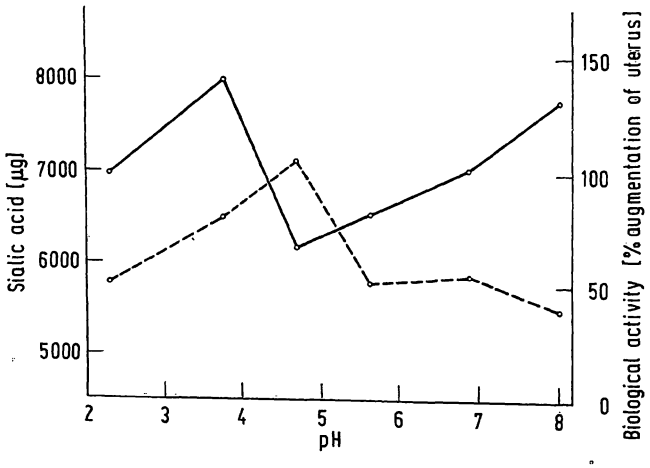

Fig. 2
Idem as Fig. 1, but considering the starting pH values of buffer

Column extraction

The method described above was applied and the results summarised in Table 3 (chemical determination) and Table 4 (biological determination), were obtained. The results are compared in Figure 3.

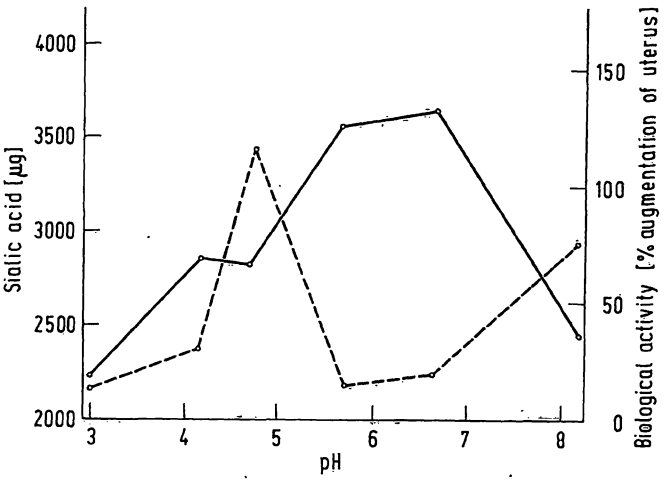

Fig. 3
Biological activity in % augmentation of the uterus of immature mice (— o —) and sialic acid content (- - - o - - -) versus pH by column extraction

Tab. 3
Column extraction, results of the chemical determination

Column No.	pH after elution	Total sialic acid content [µg]	Sialic acid content of the purified extract [µg]
I	3.0	4.560	2.272
II	4.1	4.560	2.409
III	4.7	4.560	3.451
IV	5.7	4.560	2.176
V	6.6	4.560	2.264
VI	8.1	4.560	2.945

Successive column extraction

A first extraction was performed, applying the method described above, and using a buffer solution at pH 4.7. The solid remaining on the column, was reextracted with a buffer solution at pH 8.0.

Both eluates were tested by the above biological and chemical tests.

The results are given in Table 5.

Tab. 4
Column extraction, results of the biological determination

	Zero test	I	II	III	IV	V	VI
pH		3.0	4.1	4.7	5.7	6.6	8.1
mean uterus weight/10 g animal weight [mg]	8.4 ± 0.7	9.2 ± 0.8	14.0 ± 1.7	13.8 ± 0.8	19.0 ± 2.7	19.3 ± 3.4	11.2 + 0.7
% augmentation		12.86	67.29	63.83	126.36	129.81	33.94

Tab. 5
Successive column extraction, results of the chemical and biological determinations

	Zero test	I	II
pH		4.7	4.7 followed by 8.0
sialic acid [μg]		300	300
mean uterus weight/ 10 g animal weight [μg]	5.1 ± 0.5	8.4 ± 1.8	16.1 ± 0.8
% augmentation		65.09	215.09

Discussion

The results of these experiments suggest that the pH of the extraction fluid has an influence on the nature of the compounds extracted. When we consider the results of column extraction, we can see that at pH 4 to 5 a high sialic acid content was found, corresponding to a relatively low biological activity, whereas the reverse was observed at pH 6 to 7.

In the results obtained with centrifugal extraction, we also see a high sialic acid content at pH 4 to 5, with a low biological activity. At pH 6 to 7 the opposite effect is not so apparent with respect to the final pH, whereas it can be seen to be related to the starting pH of 6.8. The percentage uterine weight augmentation is in the same range for both methods. (129% by column extraction and 101% by centrifugal extraction.)

Thus, when considering the starting pH values, both experiments show the same tendency (Figs. 2 and 3). The reason for the change in pH before and after centrifugation is not known. It could be due either to a greater neutralising effect of the extract itself or to the methodology used.

In 1971, SAXENA and RATHNAM isolated pituitary FSH by extraction at pH 6.7 and pituitary LH at pH 9.5 (9), i. e. at high pH values. The results obtained by our investigations suggest furthermore that the sialic acid contents found at lower pH values may be eliminated because they probably correspond to glycoprotein molecules lacking biological activity. In other words, this sialic acid is probably either attached to glycoprotein molecules other than gonadotrophins or it is present in free state.

By successive extraction at pH 4.7 and pH 8.0, the results suggest that the origin of sialic acid at these two pH values is quiet different. At pH 4.7 a content of 300 μg sialic acid only gives 65% augmentation of the uterus weight and after reextraction at pH 8.0, the augmentation is 215% for the same quantity of sialic acid.

The results described show that, by using an efficient extraction, the determination of gonadotropic hormones from the sialic acid content, described by VANDEN DRIESSCHE and HANS-BERTEAU, is made more accurate and gives a higher precision.

Acknowledgements

This investigation was partly aided by the "Nationaal Fonds voor Wetenschappelijk Geneeskundig Onderzoek".

Technical assistance was given by Mrs. N. BONNE, Miss M. R. STANDAERT and Mr. M. HERMANN.

References

1. KLINEFELTER, H. F., ALBRIGHT, F. & GRISWOLD, C. C. (1943), J. Clin. Endocrinol. Metab. *3*, 529—544. — 2. HANS-BERTEAU, M. J. (1960), J. Pharmacie Belgique, *15*, 179—185. — 3. VANDEN DRIESSCHE, R. & HANS-BERTEAU, M. J. (1962), Geburtshilfe, Frauenheilkunde *10*, 936—938. — 4. VANDEN DRIESSCHE, R. & HANS-BERTEAU, M. J. (1962), Rev. Iberica Endocrinologia, *IX*, 117—119. — 5. VANDEN DRIESSCHE, R. & HANS-BERTEAU, M. J. (1962), C. R. Soc. Franc. Gynecologie *5*, 1—6. — 6. HANS-BERTEAU, M. J. & VANDEN DRIESSCHE, R., Abstracts of Papers presented at the XXIII International Congress of Physiological Sciences (Tokyo 1965). — 7. SVENNERHOLM, A. (1957), Biochim. Biophys. Acta *24*, 604—611. — 8. MAC ILVAINE, T. C. (1921), J. Biol. Chem. *49*, 183—186. — 9. SAXENA, B. B. & RATHNAM, P. (1971), Protein and polypeptide Hormones (Ed. by Margoulie and Greenwood), Proceedings of the second International Symposium, Liège, p. 122—131.

E. Finné
B-1000 Brüssel
Waterloolaan 115